AD_____

Award Number: DAMD17-97-1-7296

TITLE: Cyclin C Regulation of the Stress Response and Drug Sensitivity in Breast Cancer

PRINCIPAL INVESTIGATOR: Randy Strich, Ph.D.

CONTRACTING ORGANIZATION: Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111

REPORT DATE: August 2000

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2000	Final (1 Aug 97 - 31 Jul 00)

4. TITLE AND SUBTITLE
Cyclin C Regulation of the Stress Response and Drug Sensitivity in Breast Cancer

5. FUNDING NUMBERS
DAMD17-97-1-7296

6. AUTHOR(S)
Randy Strich, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111

E-MAIL:
R_Strich@fccc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

One mechanism by which malignancies are protected from cytotoxic agents is through aberrant activation of the stress response pathway. The yeast C-type cyclin (*UME3*) is a repressor of stress response genes. To relieve this repression, the *UME3* protein (Ume3p) is rapidly destroyed when cells are exposed to several types of stress. To determine if this novel regulatory strategy is conserved, the human C-type cyclin (HcycC) levels were monitored in cell lines exposed to stress and apoptotic inducers. An epitope tagged derivative of HcycC (HcycC-HA) was constructed and stability integrated into the human breast cancer cell line MCF-7. Exponential cultures were subjected to either heat shock (42°C) or exposed to tumor necrosis factor alpha (TNFα). In both studies, HcycC levels were reduced compared to untreated controls. In addition, HcycC was rapidly destroyed when ectopically expressed in yeast subjected to heat shock. These findings are consistent with a model that down regulation of HcycC is part of the normal cellular response to stress. Moreover, these findings suggest that the regulation of C-type cyclins is conserved from yeast to man.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Cyclin/stress response/drug resistance	11
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

___ Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

___ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

X In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

X In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

___ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Randy Stucks 8/28/00

PI - Signature Date

Table of Contents

(5) Introduction.

Disseminated malignancies are commonly treated with cytotoxic agents (e. g., chemotherapy, radiation) which target the unregulated growth associated with tumors. However, many of these procedures have proven unsuccessful due in part to the acquired resistance of cancer cells to these regimens. Mounting evidence suggests that one underlying mechanism by which malignancies are protected from cytotoxic agents is through aberrant activation of a pathway generally referred to as the "stress response". This system, which is found in all organisms from procaryotes to man, elicits the expression of several conserved gene families (heat shock proteins e. g., Hsp70, Hsp27) that protect the cell from cytotoxic agents. In human breast cancer, overexpression of Hsp's has been associated with tumors that are both more invasive and/or resistant to chemotheraputic drugs. We propose to expand studies initiated in the budding yeast *S. cerevisiae* to investigate the role of the human cyclin C in regulating stress response genes in breast cancer tissues.

(6) Body

Object 1: Is HcycC down regulated as part of the normal cellular response to stress?

Task 1: Establish human cell lines stably expressing HcycC.

Stable HcycC transfectants were established in two separate cell lines, Hela and MCF7 breast cancer cells. The Hela cell line was chosen to distinguish between general effects on HcycC regulation from those specific for breast cancer cells. First, the HcycC cDNA was obtained from Dr. S. Reed, Scripts Institute (8). The cDNA was tagged with the HA epitope (15) using standard techniques. The tagged version of HcycC was inserted into the vector pCDNA3 to form pKC414 (Fig. 1) under the control of the CMV promoter which provides high, constitutive expression in many cell types.

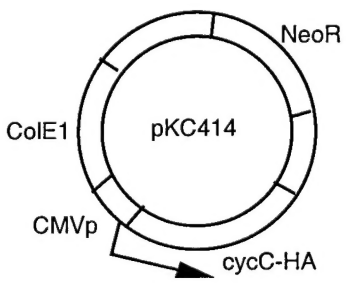

Figure 1. Map of pKC414

Stable transfectants using this construct were made in both Hela and MCF7 breast cancer cell lines by lipofection. Transfectants were selected for neomycin resistance and subclones isolated. The clones (minimum of 10) were pooled and expanded for the experiments described below. To determine the expression levels of HcycC-HA, Western blots were performed on cell extracts prepared from log-phase cells. A band corresponding to 31 kDa HcycC-HA was observed in the HcycC-HA transfected cell lines but not in the mock transfected control (Fig. 2). These results indicate that HcycC-HA has been successfully integrated into these cell lines.

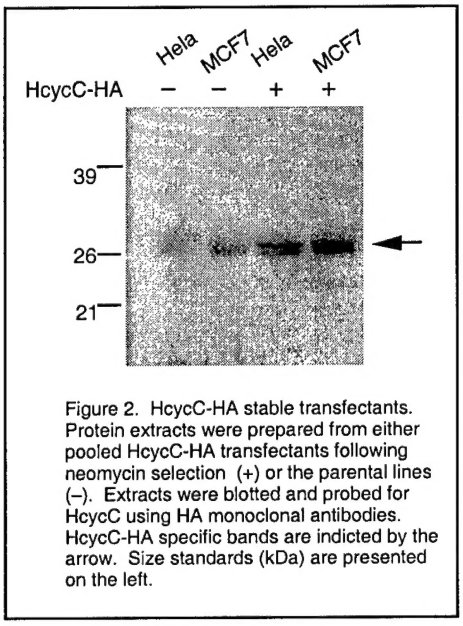

Figure 2. HcycC-HA stable transfectants. Protein extracts were prepared from either pooled HcycC-HA transfectants following neomycin selection (+) or the parental lines (–). Extracts were blotted and probed for HcycC using HA monoclonal antibodies. HcycC-HA specific bands are indicted by the arrow. Size standards (kDa) are presented on the left.

Task 2: Examine HcycC regulation in response to stress agents, heat shock, nutrient deprivation and hypoxia.

The human cyclin C is destroyed in response to stress.

The C-type cyclin subfamily shares several characteristics including association with the RNA polymerase holoenzyme and displaying a constant protein level throughout the cell cycle. To determine if the novel stress-induced destruction observed for Ume3p is also conserved, the levels of the human cyclin C (HcycC) were examined in mammalian cells exposed to stress. Antibodies raised against the endogenous HcycC (a gift from E. Lees, DNAX Inc.) were used to probe Hela cell extracts exposed to either heat shock (42°) or oxidative stress in the form of H2O2 (0.2 mM). To determine the decay kinetics of HcycC in cells exposed to stress, timecourse studies were performed. The Hela cells were grown to mid-log phase (approximately 70% confluent) prior to heat shock (42°). The cells were harvested at the indicated times and protein extracts prepared. Western blot analysis revealed that HcycC levels remained constant for 30 min. then fell to about 40% of their initial values for the two-hour duration of the experiment (Fig. 3A). The reduction in HcycC levels mirrored the increase in Hsp70 levels as indicated by Western blot analysis (Fig. 3C). The reduction of HcycC coincident with Hsp70 production mirrors our observations with Ume3p in yeast (2). Repeating these experiments with H2O2 treatment revealed decay kinetics for HcycC similar to heat shock (Fig. 3B and C). These results indicate that HcycC, similar to its yeast counterpart, is down regulated in response to stress suggesting that the C-type cyclin destruction pathway is conserved between yeast and man.

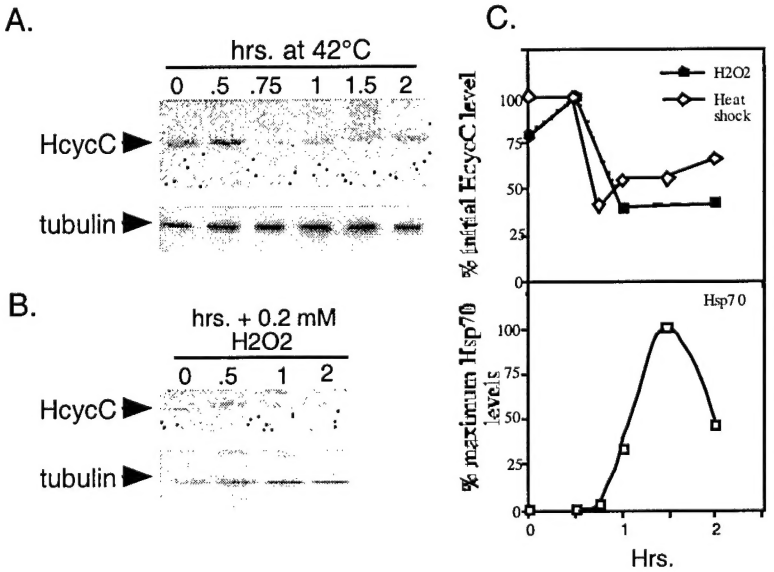

Figure 3. Analysis of HcycC levels in response to heat shock and oxidative stress. Panel A. Heat shock. Hela cells in mid-log phase growth were incubated in pre-warmed medium for the times indicated. Endogenous HcycC was identified by Western blot analysis of protein extracts using I-125 labeled secondary antibodies to allow quantitation. Panel B. Oxidative stress. The experiments described in Panel A were repeated except that 0.2 mM H2O2 was added to the medium followed by incubation at 37° for the times indicated. Panel C. Quantitation of HcycC signals. I-125 signals were quantitated by Phosphorimager (Fuji Inc.) using tubulin loading controls to standardize each sample. Hsp70 levels (lower panel) are presented for the heat shock experiment.

The next step to develop the HcycC-HA system is to determine whether the epitope tagged derivative is still destroyed in response to stress. The transfected Hela and MCF7 cell lines were grown to mid log phase, harvested, split and either left untreated or subjected to heat shock (42°C) for 2 hr. Western blots of protein extracts prepared from these samples revealed a significant reduction in HcycC-HA levels in both cell lines compared to the untreated controls (Fig. 4). These results indicate that HcycC-HA responds to stress in a manner similar to the endogenous protein. Moreover, the reduction in HcycC levels is promoter independent since both the endogenous and CMV driven HcycC proteins respond to stress in a similar manner. These results are consistent with the change in protein levels being the result of differences in stability. These studies are currently being repeated using pulse-chase experiments to confirm our hypothesis.

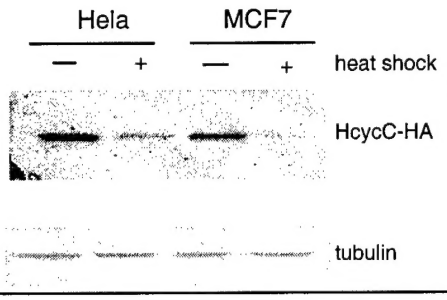

Figure 4. HcycC-HA is down regulated in response to heat shock. Pooled transfectants of the indicated cell type were subjected to heat shock for 2 hrs (+) or left untreated (-). Extracts were prepared and HcycC-HA levels were determined by Western blot analysis. Tubulin levels served as a loading control.

The final, and most stringent, test for the conservation of the stress-induced destruction pathway is the ability of the yeast system to recognize and destroy HcycC in response to stress. The HcycC-HA cDNA was placed under the control of the constitutive yeast *ADH1* promoter. Extracts prepared from yeast cultures harboring this construct contained an HA-dependent band the expected size for HcycC (33 kDa) but not in the vector control strain (data not shown). As an initial test, the HcycC-HA containing strain was subjected to heat shock (37°). Western blot analysis revealed that HcycC levels were reduced in response to heat shock (Fig. 5). As expected, no difference was observed in the HcycC mRNA accumulation from the *ADH1* promoter indicating that the reduction in HcycC-HA was most likely due to posttranscriptional mechanisms (data not shown). These findings indicate that not only is the pathway conserved, but to a remarkably high degree.

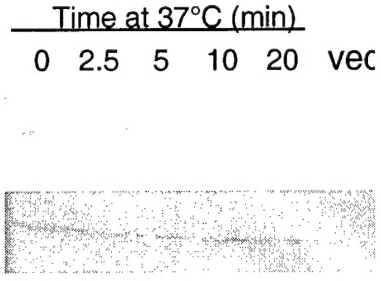

Figure 5. HcycC is destroyed in response to heat shock in yeast. HcycC-HA cDNA expressed from the yeast *ADH1* promoter was introduced into yeast via transformation. A mid-log phase culture harboring this plasmid was harvested and subjected to heat shock for the times indicated. Extracts were prepared and HcycC-HA levels were determined by Western blot analysis. Tubulin levels were used as a loading standard. The vector lane (vec) controls for non-specific binding of the HA monoclonal antibody.

Task 3: Examine HcycC regulation in response to cytotoxic agents doxorubicin, taxol, and 5-fluoro-uracil.

Due to the wide range of response kinetics observed for different stressors, an alternative approach was utilized to confirm our results from the heat shock experiments. Rather than subject the cells to stress, they were instead treated with the cytokine tumor necrosis factor alpha or TNFα. TNFα is produced by several cell types (e. g., macrophages, lymphoid cells) in response to inflammation, infection and other extracellular insults including ROS exposure (13). TNFα elicits a spectrum of cellular responses most notably fever, tumor necrosis and apoptosis (13, 14). One mechanism by which TNFα functions is through activation of the Jun N-terminal kinase/Stress Activated MAP kinase (JNK/SAPK) signal transduction cascade which represents a major stress-sensing pathway (7). In addition, TNFα appears to stimulate ROS production itself as part of its cell destruction function (10). To examine whether TNFα exposure affected HcycC levels, MCF7 cells transfected with HcycC-HA were treated with TNFα (2 μg/ml) for 24 and 48 hrs . A control flask was maintained for the same period but TNFα was omitted. Compared to the control, HcycC levels were significantly reduced in the MCF7 cell line exposed to TNFα at both the 24 and 48 hr timepoint (Fig. 6). Given the pleiotropic nature of TNFα response pathways, the precise nature of the signal leading to HcycC down regulation is not known. Although currently being confirmed by pulse chase experiments, these results are consistent with a model that HcycC is down regulated in response to stress. We are currently testing the impact of H2O2 on HcycC in both yeast and mammalian cells. Given that both cyclins are destroyed in yeast, it is possible that they share a common destruction element.

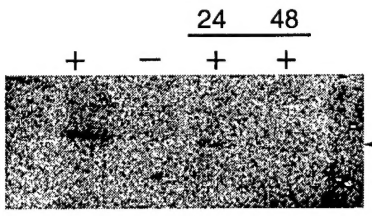

Figure 6. Regulation of cycC by TNFα. MCF7 cell line harboring cycC-HA (+) were subjected to TNFα treatment for the times indicated (hrs). The mock transfected parental line (–) serves as a control for non-specific binding of the HA Mab.

Task 4. Using directed and random mutagenesis, the cis-acting sequences the mediate HcycC destruction will be sought.

The results from these experiments indicate that the regulatory systems controlling the yeast and human C-type cyclin are well conserved. To identify cis-acting destruction elements required to mediate stress-induced degradation, we took advantage of a genetic approach in yeast that allows the selection of heat resistant derivatives of the yeast cyclin (3). Using a combination of directed and random mutagenesis, several cis-acting elements were identified (2). The two mutations identified in the genetic assay (A110V and E170K) are separated by 60 amino acids (Fig. 7). This finding is in sharp contrast to the relatively small, continuous regions (destruction box, PEST domains) required for destruction of cyclins that control cell cycle progression (9, 4, 12) or the deg1 destruction domain or degron required for Matα2 destruction (6). However, when the A110 and E170 residues are modeled onto either the human cyclin A (5) or cyclin H (1) structures, these two residues are brought together on the side of Ume3p away from Cdk binding (Fig. 7). These findings suggest the possibility that this region defines a new interactive face for cyclins.

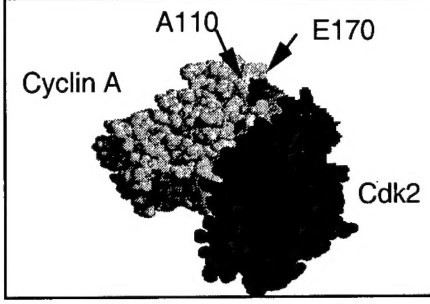

Figure 7. Ume3p stress-activated degron. The location of the A110 and E170 residues (arrows) as modeled on Cyclin A coordinates (see text for details). The position of Cdk2 is also indicated. The structure shown is based on cyclin A residues 173-432; Cdk2 residues 1-298 (full length). Modeling Ume3p sequences on the more closely related human cyclin H produced similar results.

Objective 2: Does HcycC activity effect drug sensitivity in transformed or non-transformed breast cell lines?

Task 5: Examine effect of overexpression of HcycC on drug resistance.

Two vectors were constructed that overexpressed HcycC in cos cells. These plasmids contained an SV40 origin for plasmid replication and expressed HcycC from the CMV promoter described above. The transient transfectants (and vector alone controls) were treated with three drugs used in breast cancer regimens namely doxorubicin, taxol, and 5-fluoro-uracil. No difference in viability was observed in pooled transfected exposed to either of these drugs compared to the mock transfected control (data not shown). These results indicate that overexpression of HcycC alone is insufficient to cause a significant change in drug sensitivity. However, since HcycC activates the cyclin dependent kinase Cdk8 (11), overexpressing the cyclin alone may not be functionally relevant. Therefore, experiments are ongoing in which the HcycC and Cdk8 will be simultaneously overexpressed and the drug sensitivities re-examined.

Task 6. Examine the role of HcycC in HSP gene expression.

No experiments have been completed toward this task due to grant time constraints.

Task 7: Examine drug resistance of tumor cells deleted for 6q21 with or without HcycC expression.

No experiments have been completed toward this task due to grant time contraints.

(7) KEY RESEARCH ACCOMPLISHMENTS:

- HcycC levels are reduced in response to heat shock and TNFα treatment
- A new destruction element (Stress-induced degron) has been identified that is required for the destruction of the yeast C-type cyclin in response to stress and differentiation cues.

(8) REPORTABLE OUTCOMES:

Cyclin C is destroyed in Response to stress in Breast Cancer cells. Daniel E. Egeland, Michael J. Mallory and Randy Strich. Manuscript in preparation.

Abstract: Stress-Induced Destruction of C-Type Cyclins in Yeast and Man (D-14). 1996. Yeast Genetics & Human Disease. Baltimore, MD

Abstract: Cyclin C Is Destroyed In Response To Stress In Breast Cancer Cells. 2000. Era of Hope Meeting. Atlanta, GA

Cell Lines. Two cell lines, Hela and MCF7, have been established that express a stably integrated HcycC-HA tagged construct.

(9) CONCLUSIONS:

Similar to the findings in yeast, the human HcycC levels are influenced by stress. This conclusion has been shown in two ways. First, HcycC levels are reduced in cells exposed to elevated temperatures or reactive oxygen. Second, treating cells to TNFα, a known activator of the stress response pathway, also reduces HcycC concentrations. The next step will require the actual half life of HcycC be determined using pulse chase experiments to ascertain whether the loss in cyclin levels is due to changes in stability or in translation efficiency.

(10) REFERENCES:

1. **Andersen, G., D. Busso, A. Poterszman, J. R. Hwang, J. M. Wurtz, R. Ripp, J. C. Thierry, J. M. Egly, and D. Moras.** 1997. The structure of cyclin H: common mode of kinase activation and specific features. EMBO J **16:**958-67.
2. **Cooper, K. F., M. J. Mallory, J. S. Smith, and R. Strich.** 1997. Stress and developmental regulation of the yeast C-type cyclin *UME3* (*SRB11/SSN8*). EMBO J. **16:**4665-4675.
3. **Cooper, K. F., and R. Strich.** 1999. Functional analysis of the yeast C-type cyclin Ume3p/Srb11p- RNA polymerase II holoenzyme interaction. Gene Exp **8:**43-57.
4. **Glotzer, M., A. W. Murray, and M. W. Kirschner.** 1991. Cyclin is degraded by the ubiquitin pathway. Nature **349:**132-138.
5. **Jeffrey, P. D., A. A. Russo, K. Polyak, E. Gibbs, J. Hurwitz, J. Messague, and N. P. Pavletich.** 1995. Mechanism of CDK activation revealed by the structure of cyclin A-CDK2 complex. Nature **376:**3113-320.
6. **Johnson, P. R., R. Swanson, L. Rakhilina, and M. Hochstrasser.** 1998. Degradation signal masking by heterodimerization of MATα2 and MATa1 blocks their mutual destruction by the ubiquitin-proteasome pathway. Cell **94:**217-227.
7. **Leppa, S., and D. Bohmann.** 1999. Diverse functions of JNK signaling and c-Jun in stress response and apoptosis. Oncogene **18:**6158-62.
8. **Lew, D. J., V. Dulic, and S. I. Reed.** 1991. Isolation of three novel human cyclins by rescue of G1 cyclin (Cln) function in yeast. Cell **66:**1197-1206.
9. **Murray, A. W., and M. W. Kirschner.** 1989. Cyclin synthesis drives the early embrionic cell cycle. Nature **339:**275-286.
10. **Obrador, E., J. Navarro, J. Mompo, M. Asensi, J. A. Pellicer, and J. M. Estrela.** 1998. Regulation of tumour cell sensitivity to TNF-induced oxidative stress and cytotoxicity: role of glutathione. Biofactors **8:**23-6.

11. **Rickert, P., W. Seghezzi, F. Shanahan, H. Cho, and E. Lees.** 1996. Cyclin C/CDK8 is a novel CTD kinase associated with RNA polymerase II. Oncogene **12:**2631-2640.

12. **Salama, S. R., K. B. Hendricks, and J. Thorner.** 1994. G1 cyclin degradation: the PEST motif of yeast Cln2 is necessary, but not sufficient, for rapid protein turnover. Mol. Cell. Biol. **14:**7953-7966.

13. **Tracey, K. J., and A. Cerami.** 1993. Tumor necrosis factor, other cytokines and disease. Annu Rev Cell Biol **9:**317-43.

14. **Vandenabeele, P., W. Declercq, B. Vanhaesebroeck, J. Grooten, and W. Fiers.** 1995. Both TNF receptors are required for TNF-mediated induction of apoptosis in PC60 cells. J Immunol **154:**2904-13.

15. **Wilson, I. A., H. L. Niman, R. A. Houghten, A. R. Charenson, M. L. Connolly, and R. A. Lerner.** 1984. The structure of an antigenic determinant in a protein. Cell **37:**767-778.

PERSONNEL:

Daniel Egland, Ph.D. Postdoctoral Associate